guanosine at position 65, and for A* as *O*-β-D-ribofuranosyl-(1″–2′)-adenosine in position 64. We elucidated that the final structure for A* at position 64 in yeast initiator $tRNA^{Met}$ was established as an *O*-ribofuranosyl-(1″–2′)-adenosine-5″-phosphate linked by a 3′5′-phosphodiester bond to G at the position 65.

An unknown U* nucleoside in position 34 isolated from yeast mitochondrial $tRNA^{Leu}$ was characterized as $cmnm^5U$ by HPLC-UV-MS.

Also, we have confirmed m^3U, an unknown modified uridine, in the 16S colicin fragment from *E. coli* rRNA, and report the structural characterization of a catabolite in canine urine as 5-hydroxymethycytidine (om^5C).

This report describes the 'research tools' we have developed and are using in analytical characterization of modified nucleosides and dinucleosides in RNAs and which will be of value to others in molecular biology investigations.

A good clinical correlation was observed in patient management using four cancer modified nucleoside biomarkers in following the course of disease and treatment.

See also: **II / Chromatography: Liquid:** Detectors: Ultraviolet and Visible Detection; Mechanisms: Reversed Phases.

DropBooks

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Thin-Layer (Planar) Chromatography

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Introduction

Thin-layer (TLC; or planar chromatography) is well suited to the separation of nucleic acids. One of the

most important chromatographic systems for nucleic acids – ion exchange chromatography – received great impetus with the development of poly(ethylene imine)-HCl prepared cellulose (PEI), which became available in the early 1960s. The studies that followed laid the foundation for the analytical and preparative TLC of nucleic acids. Many types of plate are presently available for TLC, but most reports are limited to PEI-cellulose, ODS (octadecylsilica), and silica gel in simple one-dimensional systems.

Gel electrophoresis has diminished the need for TLC of large oligonucleotides and the inability to have stable thick (2 mm or more) chromatographic plates has diminished the development of preparative TLC. High performance liquid chromatography (HPLC) has become important for smaller oligomer separations, and especially important for preparative chromatography. TLC and HPLC together can serve for initial investigations, but usually require further analytical instrumentation or chemical characterization. Coupled UV and Fourier transform infrared (FTIR) have added to the power of chemical characterization by HPLC and these systems are now becoming available in TLC. HPLC, however, is limited when employing highly radioactive molecules as extensive cleaning and decontamination of the whole HPLC system is required. In the hands of expert experimentalists TLC offers outstanding flexibility that matches HPLC, with less labour and cost.

Sorbents

Considerations for sorbents are: physical and chemical properties, pore diameter, pore volume, surface area, particle size distribution and mean size. Adsorption is the main mode of chromatography employed. Weak physical interactions in TLC include van der Waals forces, dipole–dipole forces and hydrogen bonding. Cellulose ion exchange further employs polyethyleneimine $(-CH_2-CH_2-NH)_n$ for more specific separations. Typically, polar solvents are employed for polar solutes with hydrophobic phases. Solvents are based on an eluotropic classification with elution power increasing with polarity. The speed of elution also depends on the viscosity of the eluent.

Cellulose is used when ion exchange properties are not needed. It is most often used for the separation of sugars, amino acids and similar compounds. A popular sorbent for the separation of nucleic acid derivatives, it readily separates pyrimidines (higher R_F) from purines. Commercial grade microcrystalline cellulose (Avicel) has been used for the retention of guanine (base or nucleoside) in either acidic (HCl; formic acid) or basic (ammonia) solvents.

Diethylaminoethylcellulose (DEAE) has the functional group incorporated into the paper. It is an anion exchanger that is generally used to separate proteins and enzymes and similar materials, but is also used for nucleic acids, nucleotides, deoxynucleotides and nucleosides. Separation on DEAE-cellulose is not as sharp as on PEI-cellulose, but there is a considerable amount of information on the separation of nucleic acids on these layers.

There are many published tables that contrast TLC separations with various solvents and demonstrate the utility of cellulose in the relative retention of amino groups, regardless of purine/pyrimidine structure, in either acid (HCl, isobutyric acid) or ammonium hydroxide mixtures. The presence of ammonium carbonate (and to a lesser extent formate) affects purine/pyrimidine separations, with R_F values greater for pyrimidines.

ECTEOLA is the abbreviation for the epichlorohydrin and triethanolamine groups that are combined with cellulose. DEAE-cellulose and ECTEOLA-cellulose layers have about the same ability to resolve nucleic acid derivatives. ECTEOLA-cellulose is especially useful for the separation of nucleic acids, nucleotides and nucleosides as an anion exchanger and is also good for the rapid separation of pyrimidines from purines.

ITLC (instant TLC) plates are glass microfibre sheets. The addition of silicic acid or silica gel gives the additional designation of SA or SG, respectively. Multiple solvent systems used with these plates allow the retention of adenine and its associated structures.

Silica gel has been used extensively, although it was not used in the early development of the TLC of nucleic acids. It is also used for the separation of amino acids and proteins. It is especially advantageous in separating pyrimidine from purines.

G is the designation for $CaSO_4$ binder (gypsum). Silica-G has been used to resolve pyridine nucleotides, uridine diphosphate (UDP) derivatives of hexosamines and acetylhexosamines Silica-G is used for preparation of larger quantities of bases, nucleosides and many of their derivatives.

Reversed-phase (RP; ODS or C_{18}) performs essentially as silica gel. The opportunity of developing a strategy on RP-TLC and transferring it to a similar HPLC system is possible, but not always successful. The utilization for TLC of commonly available premixed HPLC solvents (methanol, acetonitrile, tetrahydrofuran, phosphates) is very convenient and allows information to be obtained quickly. RP-TLC, as opposed to its HPLC counterpart, cannot be as easily employed over a wide range of pH values.

Much of the knowledge of PEI-cellulose has come from the work of Randerath. This material has been

extensively studied and used in the separation of nucleic acid bases, nucleosides and nucleotides, with good separation and resolution. It has also been used for the separation of RNA and DNA hydrolysates, and for large scale preparations among other applications. It remains the most versatile paper for the separation of deoxynucleotide monophosphates (dNMP).

High performance (HP) TLC is constantly undergoing improvements; it offers smaller layers, more uniform and smaller sorbent particles, thinner layers and faster development. HPTLC can be utilized for nucleic acid identification, but is not commonly used. Typically, HPTLC offers quantities of product that are too small for identification by GC, FTIR or NMR.

Preparative TLC is a rapid technique where the analyte is streaked across a plate, and separation commences on a layer 1–5 mm thick. The nucleic acid of interest is scraped off the plate and eluted accordingly. Papers for centrifugal layer chromatography offer an alternative preparative technique. Chiralplates have had excellent results in separating enantiomers and halogenated compounds, and can also play a role in separation of nucleic acids.

Solvents

These are discussed in detail in the publications given in the Further Reading section. Tactically, an initial screen of unknowns or products on TLC is carried out as a preparation for the development of an HPLC method. This is helpful, and the rule of thumb is that 20 one-dimensional TLC runs with different solvents will determine the best eluent.

Directionality, including ascending, descending, two- and multi-dimensional, circular and drip chromatography have all been employed either to improve separations or to increase sample throughput. Automated anti-circular TLC systems where the solvent is applied at the periphery and flows inward toward the centre offer improved ability to examine fractions with R_F values near 1.

Results are essentially empirical, with advantages for most techniques based on analysis time for a specific set of analytes. Excellent reproducibility and success has been obtained with two- or multi-dimensional TLC, which greatly enhances the number of theoretical plates available, and hence the ultimate separation. Significant progress in gradient TLC will also impact on nucleic acid separations.

TLC has great flexibility – concentration, viscosity, polarity, pH, ionic strength, composition of gas phase and temperature are all important and controllable. Educated trial and error is not inappropriate to develop initial TLC characterization of analytes. The strategy of many separation techniques emphasizes the chemical differential migration, e.g. hydroxyl, ammonium groups, of the various dNMPs with selective retention. The solvent affects all components of a mixture equally as a nonselective driving force. Further resolution of dNMPs from DNA can be accomplished by selective removal of particular compounds, or groups of compounds; to emphasize or diminish a specific dNMP we would consider competing with an analogue, e.g. deaza-dGMP for dGMP adducts, or depurinating to emphasize pyrimidines (see **Figures 1** and **2**). This strategy allows us to maintain simplicity in the solvent system.

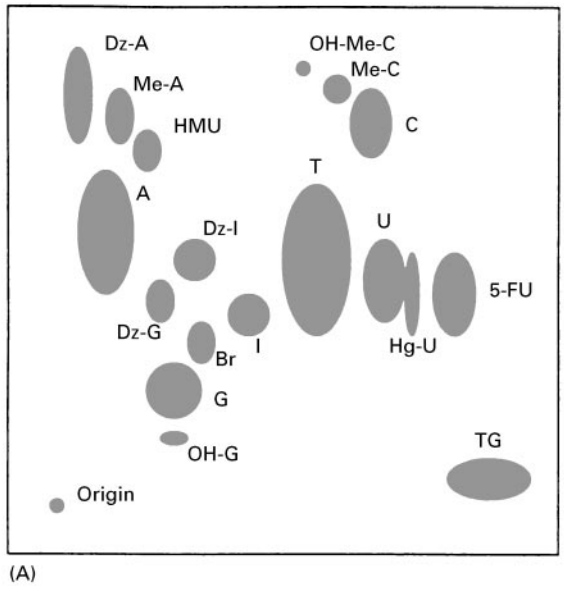
In optimizing planar chromatography, computer programs exist that are very helpful in developing choices for solvents. Demixing remains a major problem in predicting retention and the ultimate experimental outcome. Again, 20 chromatograms are sufficient to define experimental variables for optimum resolution. Solvent selectivity has been discussed in terms of proton donation, acceptance or dipole interactions. Many mixtures of solvents exist, yet a reliable few can serve almost all purposes. We have listed the common solvents for TLC as a guide in **Table 1**.

DropBooks

Uniformity of Techniques

A general problem with TLC is the paucity of uniform guidelines that can direct investigators in different laboratories. Any serious investigator in TLC systems must establish rigorous and reproducible techniques. Few papers give in detail all the parameters necessary to reproduce successfully experimental TLC protocols. Much of this is pragmatic, especially given simple unidimensional systems where only one known chemical, with established controls, is confirmed. Most typically, these are pharmaceutical-based studies, which simply confirm one known pharmaceutical that conforms to the available control.

Success in characterizing true unknowns in complicated bi- or multidirectional systems (two-dimensional TLC) requires the publication and listing of uniform criteria. Attempts have been made to validate TLC techniques by directing attention to a number of concerns including R_F reproducibility, the role of the mobile phase, the stationary phase, the quality and quantification of zones, the method of elution and the estimation of spots. It is suggested that published TLC papers should attempt a more uniform approach to stating clearly experimental materials, methods and conditions. In view of the need for interlaboratory reproducibility, listed below are the



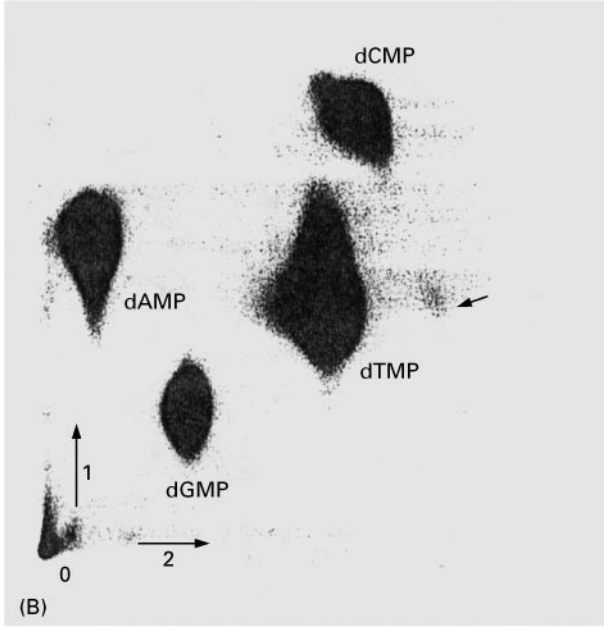


Figure 1 (A) Stylized representation of nucleic acid separations. (B) Normal enzymatic and ^{32}P-labelled digest of placental DNA.

chromatographic conditions necessary for the successful separation of dNMPs on PEI-cellulose:

1. Solvents (composition): first dimension, acetic acid (1.0 mol L^{-1}, pH 3.5 with NaOH); second dimension, 5.6 mol L^{-1} $(NH_4)_2SO_2$, 0.12 mol L^{-1} Na_2EDTA, 0.035 mol L^{-1} $(NH_4)HSO_4$ to pH 4.0. Stable over 2 weeks.
2. Layer (brand, grade): PEI-cellulose, Sigma.
   - Size, geometry: square, 200 mm × 200 mm.
   - Method of storage: refrigerator at 4°C.
   - Preparation: no pre-run; constant room temperature and humidity.
   - Treatment: cool air-dried (dehumidified) during spotting.
   - Heterogeneity (R_F lower with thicker paper): > 1–3% variation over each TLC.
3. Developing tank (make, size): Sigma Inc; 275 mm × 275 mm × 75 mm with lid.
4. Application amount: 1.0–10.0 μL (or 20 000–100 000 CPM (counts per minute)).
5. Drying (origin, plate, after first dimension): at 1 cm, 1 cm x, y axis; cold dryer.
6. Direction of development: ascending, both dimensions.
7. Distance of origin from solvent reservoir (closer for higher R_F): 1.0 cm.
8. Depth of immersion: 5 mm.
9. Volume of solvent in reservoir: 15 mL.
10. Duration of development (h): first dimension, 4 h; second dimension, 15 h.
11. Temperature: 17°C; 50–60% humidity constant.
12. Equilibrium humidity in tank: complete prior to TLC.
13. Character of solvent front: observe as regular, linear.

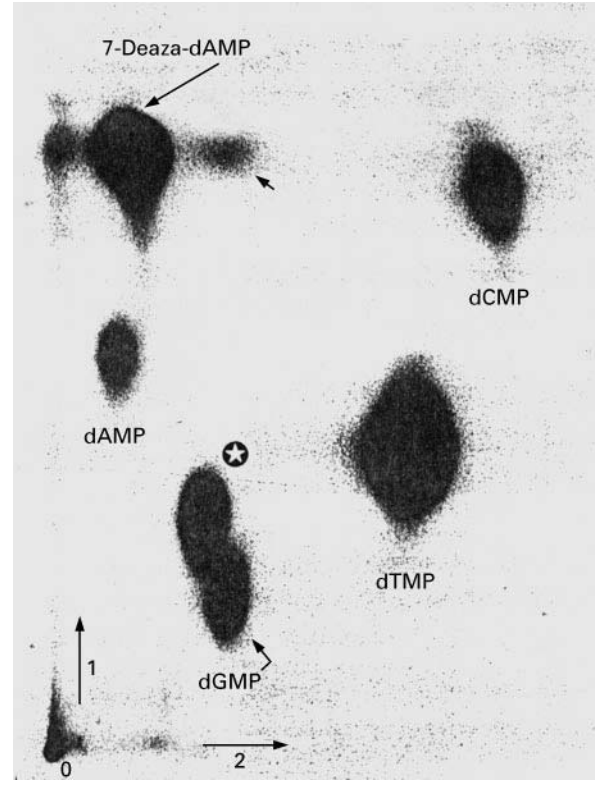


Figure 2 DNA analogue-labelled digest: deaza-dAMP replacing dAMP.

14. Comparison of R_F versus R_X: consistency of chemical migration versus relative standard – less than 3% variability. All R_F values given as 'R_X' with x, y coordinates. NB: Conversion of R_X to R_F requires all numbers divided by 19 cm. (If R_F values are given these are usually multiplied by $100 = hR_F$.)

Most parameters in TLC are quantifiable, and all quantitative information should be listed. A greater attempt by investigators, reviewers and editors to adhere to these standards will increase both the likelihood of chromatographic success, its reproducibility, and sensitivity.

Sensitivity

In our 2D-TLC system for dNMPs we attempt to discover and analyse altered nucleic acids (adducts) or synthetic nucleic acids used as pharmaceuticals (analogues). The technique can ultimately detect one radioactive adduct per 10^8 nucleotides, which is as sensitive as any analytical system available. At this stage we radiolabel 0.2 μg of DNA with ^{32}P to 6.0×10^6 DPM (disintegrations per minute). We assay from 2.0×10^4 to 1.0×10^6 DPM and can reliably detect as few as 50 DPM over background. This allows a mathematical minimum detection of one adduct per 10^5–10^8 nucleotides. Yet, many unique analytes can be detected at up to one per 10^8–10^{10} dNMP (as few as 25 DPM above background). An example of detectability can be seen by the complicated pattern of adducts formed by DNA in buffer remaining refrigerated for a prolonged period and obvious DNA reactivity with water and oxygen as noted by the proliferation of adducts at 72 h autoradiography. Furthermore, we have detected adduct incorporation when we have altered and 'contaminated' the dNMP reaction mixture pool with less than 1 nmol of a foreign dNMP during enzymatic incorporation (see **Table 2**). These lower values are within the range for detecting modifications by environmental, drug and ageing processes, e.g. methylated or deaminated dNMPs.

Reliability

Control dNMPs and DNA are run with every batch of samples. Experience with this technique shows that variations of R_F values ranging from 1 to 5% can be achieved in over 2000 analyses. The Ambis (computerized radioactive scanning of TLC) statistically correlates consistently with laser densitometry, but mean values can vary in densitometry by ±6.5% overall. There are also qualitative differences between densitometry and scintillation counting, specifically where densitometry is unable to account for all the 'spots' that it detects as a 'smear', though the human eye can easily distinguish borders, zones and spots. However, the Ambis is more successful in quantifying smear areas by counting smear CPM. A statistical analysis of Ambis data versus densitometry provides a correlation coefficient of $0.93, p < 0.001, n = 23$ pairs, providing the formula: Ambis DPM = 62.4 (mm^2 area from densitometry) −17 410. The Ambis is more successful in detecting dUMP (see **Figure 3**) and other less discrete dNMPs than densitometry. Yet densitometry shows up borders between migration patterns of close dNMPs better, especially methylated dNMPs. Other variations in CPM reflect quenching of radioactivity from the TLC plates. At low DPM, quenching blocks 90% of counts detectable, but at high DPM, quenching blocks only 50% of counts. These differences are mathematically quantifiable, and the formulae generated have high predictability. TLC data must be presented as quantitative and statistical values to further increase reliability of techniques, and correlate inter laboratory discrepancies.

Analyte Identification

The major ability to both elute and identify any nucleic acid resides in its functional groups and heterocyclic rings. Any approach to identification of unknown or modified nucleic acids should begin with characterizing functional groups, and subsequently using this information to improve separation. Furthermore, functional groups offer sites of chemical alteration and simple bench-top techniques, can confirm structure. tRNA has served as the primary impetus for developing accurate and reproducible techniques to separate methylated nucleosides. It is not possible to summarize in this article the literature that exists on the chemistry of nucleic acids.

In consideration of both choice of TLC paper and likely nucleic acid identification, a few observations apply. In general hydrophobic modifications and methylation decrease R_F, hydrophilic modifications, e.g. succinylations, increase R_F. Low versus high negative nucleic acid charges are alterable and dependent on TLC and solvent system. Other strategies may be first to react chemically with nucleic acids prior to chromatography.

Sugars such as pentose ring riboses and deoxyriboses react readily. The sugars are uncharged at physiological pH, and lose a proton at pH 12. The major advantage of the phosphodiester bond is that it is cleaved with extreme acid or alkali. The charge and number of the phosphates ultimately confer their

DropBooks

Table 1 Nucleic acid and nucleotides R_F values using various TLC plates and solvents

	P1	*P1*	*P2*	*P2*	*P2*	*P2*	*P2*	*P3*	*P3*	*P3*	*P3*	*P3*	*P4*	*P5*	*P5*	*P6*	*P7*	*P8*	*P8*	*P8*	*P8*	*P9*
Nucleic acid	S1	S5	S8	S9	S10	S11	S12	S2	S3	S4	S6	S7	S3	S3	S4	S1	H20	S13	S14	S15	S16	S1
$R_F \times 100$	T1	T3	T6	T6	T6	T6	T6	T2	T2	T2	T5	T5	T2	T2	T2	T1	T4	T2	T2	T2	T2	T1
Adenine	30	–	31	88	36	40	10	98	20	14	55	98	33	44	13	29	8	75	85	54	51	38
Guanine	37	–	16	50	13	25	15	73	10	23	52	98	31	40	0	33	18	00	00	00	44	38
Uracil	72	–	70	57	35	47	54	74	26	62	113	102	66	75	68	73	86	58	72	54	83	75
Cytosine	–	–	41	59	31	59	59	92	26	50	136	138	80	90	56	–	40	–	–	–	–	–
Thymine	–	–	80	65	57	57	44	83	41	52	–	–	74	85	54	–	81	–	–	–	–	–
Adenosine	53	77	23	–	–	41	–	91~	68	–	92	99	58	62	–	56	8	65	80	50	65	75
Guanosine	58	–	22	50	9	26	27	59~	13	–	84	98	31*	39*	–	50	77	22	17	17	74	80
2-Deoxyadenosine	–	–	27	82	37	42	9	97~	66	–	–	–	70	70		–	–	–	–	–	–	–
2-Deoxyguanosine	–	–	14	59	17	36	23	73~	12	–	–	–	40	50	–	–	–	–	–	–	–	–
2-Deoxycytidine	–	–	50	65	35	–	47	90~	93	–	–	–	82	90	–	–	–	–	–	–	–	–
Cytidine	80	55	36	65	27	–	52	78~	96	–	139	138	79	94	–	82	55	30	37	23	90	76
Thymidine	–	–	88	66	52	55	37	81~	49	–	–	–	81	90	–	–	–	56	75	51	78	–
Uridine	81	39	62	–	–	–	–	63~	30	–	130	102	68	87	–	84	93	39	50	37	90	85
Deoxyuridine	–	–	77	61	33	47	45	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Reference	A	1A	3A	6A	6A	6A	6A	2A	2A	2A	5A	5A	2A	2A	2A	1A	4A	7A	7A	7A	7A	1
Nucleotides			P3	P3	P3	P3	P3	P3	P3	P4	P5	P6	P6	P6	P10	P10	P10	P11	P11	P11	P11	
Solvent			S20	S17	S21	S22	S23	S31	S32	S19	S19	S16	S17	S18	S28	S29	S30	S24	S25	S26	S27	
Technique			T9	T10	T10	T10	T10	T13*	T13*	T9	T9	T7	T7	T8	T12	T12	T12	T11	T11	T11	T11	
Adenosine-5′-monophosphate			–	45	65	–	–	–	–	–	–	57	26	–	–	–	–	38	35	52	41	
Adenosine monophosphate			–	–	–	–	–	76	94	–	–	–	–	42	22	47	54	–	–	–	–	
Adenosine-3′-monophosphate			63	–	–	–	–	–	–	4	24	48	–	–	–	–	–	–	–	–	28	
Adenosine-2′-monophosphate			63	–	–	–	–	–	–	4	32	–	–	–	–	–	–	–	–	–	35	
Adenosine diphosphate			–	24	48	68	–	65	26	–	–	36	8	36	4	27	46	26	17	29	48	
Adenosine triphosphate			–	6	11	20	56	63	6	–	–	21	–	32	0	16	37	16	8	16	56	
Cytidine-5′-monophosphate			–	46	65	–	–	–	–	–	–	74	31	–	–	–	–	34	30	48	–	
Cytidine-3′-monophosphate			51	–	–	–	–	–	–	5	50	71	–	–	–	–	–	–	–	–	–	
Cytidine-2′-monophosphate			55	–	–	–	–	–	–	9	61	–	–	–	–	–	–	–	–	–	–	
Cytidine diphosphate			–	31	53	–	–	89	64	–	–	51	11	–	7	38	56	22	13	27	–	
Cytidine triphosphate			–	9	13	31	64	86	18	–	–	34	–	–	0	21	47	13	7	13	–	
Guanosine-5′-monophosphate			–	–	–	–	–	–	–	–	–	55	14	–	–	–	–	–	–	–	–	
Guanosine-3′-monophosphate			–	–	–	–	–	–	–	–	–	44	–	–	–	–	–	–	–	–	49	
Guanosine diphosphate			–	–	–	–	–	50	14	–	–	37	3	–	0	28	49	–	–	–	–	
Guanosine triphosphate			–	–	–	–	–	50	2	–	–	17	–	–	0	15	35	–	–	–	–	
Thymidine-5′-monophosphate			47	–	–	–	–	–	–	5	48	–	–	–	–	–	–	–	–	–	–	
Thymidine monophosphate			–	–	–	–	–	–	–	–	–	–	–	110	–	0	0	–	–	–	–	
Uridine-5′-monophosphate			–	–	–	–	–	–	–	–	–	80	13	–	–	–	–	37	30	47	–	
Uridine-monophosphate			–	–	–	–	–	92	73	–	–	–	–	53	44	74	76	–	–	–	–	
Uridine-3′-monophosphate			36	–	–	–	–	–	–	2	42	75	–	–	–	–	–	–	–	–	–	

Uridine-2′-monophosphate	36	–	–	–	–	–	–	2	48	–	–	–	–	–	–	–	–	–	–
Uridine diphosphate	–	7	15	25	–	86	18	–	–	63	0	42	9	55	69	25	14	26	–
Uridine triphosphate	–	4	4	8	18	81	6	–	–	44	–	37	4	38	58	17	8	13	–

Papers:

P1 = cellulose celite w/5% starch binders; P2 = commercial grade microcrystalline cellulose (avicel); P3 = DEAE-CELLULOSE; P4 = DEAE-CELLULOSE with 5% $CaSO_4$; P5 = DEAE-CELLULOSE with 10% $CaSO_4$; P6 = ECTEOLA; P7 = ITLC-SA; P8 = polyamide; P9 = silica; P10 = ITLC-SAF impregnated with 1% polyethylenimine techniques; P11 = MN-300 and MN-300 G (with plaster of Paris) cellulose.

Techniques for nucleic acids:

T1 = ascending development, 10 cm in 45 min; T2 = wang method; T3 = 12 cm development distance; T4 = ascending, saturated chamber; T5 = gradient elution, two-dimensional chromatography; T6 = room temperature 18–22°C, for two-dimensional, 11–13 cm, dry at 40°C, then 8–9 cm in the second direction.

Techniques for nucleotides:

T7 = 15 minutes development time, 8–10 cm; T8 = ECTEOLA-cellulose sprayed with 0.01 ammonium tetraborate (pH 9.0) and dried with 17 cm run, 2 h/closed tank; * = R_f rel to inorg. phosphate; T9 = closed tanks or open trays when two-dimensional development, 6 inches; T10 = 5–10 min; T11 = 50–60 min; T12 = stepwise development, S28-45 s, S29-6 min and S30 to 13 cm; T13 = gradient elution, two-dimensional; * = ref. rel to CMP.

Solvents:

S1 = H_2O; S2 = isobutyric acid/conc. ammonium hydroxide/water (33 : 1 : 16); ~ = S2 with 10% $CaSO_4$; S3 = 0.005 N HCl; S4 = saturated ammonium sulfate/1 N sodium acetate/isopropanol (40 : 9 : 1); S5 = isopropanol/water (9 : 1); S6 = 75 mL of 1.0 mol L^{-1} NH_4HCO_3 in one chamber and 85 mL of 0.15 mol L^{-1} NH_4HCO_3 in the second chamber; S7 = 75 mL of 2.0 mol L^{-1} ammonium formate, pH 4.2, in one chamber and 85 mL of 0.2 mol L^{-1} ammonium formate, pH 2.8, in the second chamber; S8 = isopropanol/HCl/H_2O (65 : 16.7 : 18.3 v/v); S9 = isobutyric acid/H_2O/25% NH_4OH (400 : 208 : 0.4 v/v); S10 = isopropanol/H_2O/concentrated (28%) NH_4OH (85 : 15 : 1.3); S11 = *n*-butanol/H_2O/formic acid (77 : 13 : 10); S12 = saturated $(NH_4)_2SO_4$/1 mol L^{-1} sodium acetate/isopropanol (80 : 18 : 2); S13 = heptane/butanol/acetic acid (4 : 4 : 1), development time for 8 cm, 60 min; S14 = carbon tetrachloride/acetic acid/acetone (4 : 1 : 4); S15 = toluene/pyridine/ethylenechlorohydrin/0.8 N NH_4OH (5 : 1 : 5 : 3 : 3 v/v); S16 = 0.15 mol L^{-1} sodium chloride; S17 = 0.01 N HCl; S18 = 95% ethanol/0.1 mol L^{-1} ammonium tetraborate pH; S19 = 0.005 N HCl; S20 = isobutyric acid/concentrated ammonium hydroxide/water (33 : 1 : 16); S21 = 0.02 N HCl; S22 = 0.03 N HCl; S23 = 0.4 N HCl; S24 = *n*-butanol/acetone/acetic acid/5% ammonium hydroxide/water (4.5 : 1.5 : 1.5 : 1); S25 = *n*-butanol/acetone/acetic acid/5% ammonium hydroxide/water (3.5 : 2.5 : 1.5 : 1.5 : 1); S26 = *t*-amyl alcohol/alcohol/formic acid/water (3 : 2 : 1); S27 = saturated ammonium sulfate/1 mol L^{-1} sodium acetate/isopropanol (80 : 18 : 2); S28 = 2 mol L^{-1} LiCl; S29 = 1.0 mol L^{-1} LiCl; S30 = 1.6 mol L^{-1} LiCl; S31 = 75 mL of 1.0 mol L^{-1} NH_4HCO_3 in one chamber and 85 mL of 0.15 mol L^{-1} NH_4HCO_3 in the second chamber; S32 = 75 mL of 2.0 mol L^{-1} ammonium formate, pH = 4.2 in one chamber and 85 mL of 0.2 N ammonium formate, pH = 2.8 in the second chamber.

Chromobooks

Table 2 Purine/pyrimidine detection schema

Moiety	*Reagent/reaction*	*Result: nucleic acids*
Nucleoside mono-, di- and triphosphates	Ammonium molybdate–perchloric acid	Blue spots
Caffeine	Chloramine-ammonia	Rose-red coloured spots
Xanthine derivatives	Chlorine-ammonia	Xanthine derivatives – violet
Deoxyribonucleosides, deoxyribo-, mono-, di- and triphosphates	Cysteine–sulfuric acid	Purines – green fluorescence
Purines	Eosine–mercury chloride	Purines – red violet best seen under UV light
Purines	Mercuric acetate–diphenyl carbazone	'Shadows' on violet background (circle after appearance not stable)
Purines and pyrimidine bases	Mercuric nitrate–ammonium sulfide	Black spots
Oxidized pyridine nucleotides, adenine-containing compounds	Potassium cyanide	Oxidized pyridine nucleotides – fluorescent zones; adenine-containing compounds
Adenine, guanine, hypoxanthine, xanthine and derivatives from cytosine and derivatives from chloride, bromide and iodide from histidine	Silver nitrate–bromophenol blue	Adenine, guanine, hypoxanthine, xanthine, and derivatives – blue spots Cytosine and derivatives – royal blue; chloride, bromide, iodide – violet
Purines	Silver nitrate–sodium dichromate	Red spots
Sulfur derivatives of purines and pyrimidines	Thiocarbamide reaction	Green or blue spots
Guanine and xanthine and compounds from other naturally occuring purine and pyrimidine derivatives (except urate)	UV light + exposure to HCl	Dark spots against fluorescence of paper (except uric acid) guanine and xanthine exposed to HCl-strong fluorescence

mobility on chromatography. The monoester phosphate has two ionizable OH groups, and is in relative equilibrium at physiological pH.

Studies have been carried out on the lipophilic characteristics of xanthine and adenosine derivatives. These are potentially important for large classes of drugs, including chloroadenosine. Lipophilic characteristics can be studied with silicone-coated or C_{18} TLC plates. Methanol/phosphate buffer, pH 7, with a methanol content ranging from 30 to 80% have been used. Equations have been obtained to allow maximum allowable separations of 44 purines.

Separation of hydroxy-2′-deoxyguanosine-3′-monophosphate is carried out in 1.5 mol L^{-1} ammonium formate (pH 3.5), and then 0.4 mol L^{-1} ammonium sulfate. Though good separation of C_8-hydroxy-dGMP is obtained, most dNMPs remain in the midline, with significant artefacts in the second dimension. Aside from ageing, metabolism of DES (the hormonal drug diethylstilbesterol) also forms C_8-hydroxy-dGMP.

Novel separations of anomeric α (pharmacological) purines can be carried out on copper acetate Chiralplates with methanol/water/ACN and visualization under UV light.

Others use silica gel separations of noncyclic radioactive [^{3}H] adenosine as neuromodulators, and only use one-dimensional separation. The solvents are various mixtures of butanol/ammonia/water/acetic acid. Separation takes 3–4 h. Typical separations unequivocally demonstrate cAMP, inosine, adenosine and adenine. UV sensitivity is down to 5 nmol. Plant cytokinins (adenine) are separated on silica gel in ethanol/ammonium borate, butanol/ammonia or butanol/water. Measured ATP is obtainable from degraded meat via 5% cold perchloric acid on silica gel and isobutanol/amyl alcohol/ethoxyethanol/ammonia/water (mature solvents for 48 h).

Guanine can be separated from other nucleic acids on PEI-cellulose with triethylammonium bicarbonate (TEAB) 0.5 mol L^{-1} pH 7.6. Good separation of cyclics, phosphates and nucleosides is evident. Cyclic purines are separated by PEI with ammonium acetate/hydroxide/ethanol eluent at pH 9.0 in one dimension, ascending from triphosphates to nucleobases. Cyclic purines are also separated with PEI in 0.4 mol L^{-1} acetic acid, then 0.125 mol L^{-1} LiCl. GTP is separated with PEI and Luciferase, water and then 1.4 mol L^{-1} LiCl for 50 min. Assay is by scintillation counter.

Alkylated deoxyuracil separation is carried out with RPTLC in methanol/propanol/water/dichloroethane. Water/ethanol has greatest effects on longer chains. Here TLC demonstrates quantitative structure–activity relationships (QSAR).

Thymine dimer separation has been successful on silica gel with one-dimensional chloroform/methanol/water and two-dimensional ethyl acetate/propanol/water, then sprayed with cysteine/sulfuric acid. Separation is evident, but almost all in midline.

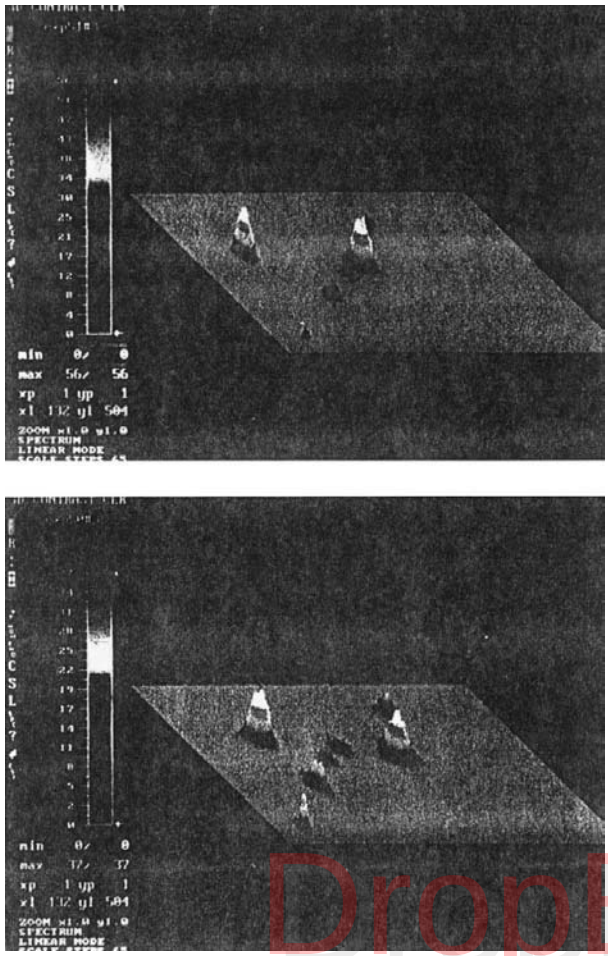


Figure 3 Three-dimensional ^{32}P computer reconstruction of DNA digest and DNA digest with chemically introduced dUMP.

Thymine dimers are also separable via cellulose and *n*-butanol/water, and 2D-ammonium sulfate/sodium acetate/propanol. Adhesive tape can be used to remove cellulose for scintillation counting.

'Bench-top' chemistry can be successfully employed for chemical identification of nucleic acids with derivatization by dimethylaminonaphthalene-5-sulfonyl chloride (DANS-Cl) formic acid (6%), acetate/ethanol/ammonium hydroxide, or ethyl acetate/ethanol/ammonium hydroxide used on a polyamide sheet. Also borohydride is used in post-labelling reduction [^{3}H].

Halogenated uracils can be separated on silica HPTLC plates. Solvents are chloroform/ethanol/water $\pm$ acetate. As many as 27 pyrimidine analogues have been separated. Cellulose TLC and various combinations of butanol/ammonia/ethyl acetate/formic acid/sodium phosphate/propanol/isoamyl alcohol were used. New analogues are regularly discovered by 2D-TLC with PEI in isobutyric acid/water/ammonium hydroxide (first dimension) and ammonium sulfate/isopropanol/sodium acetate (second dimension). Among these is 'pdJ', a nucleotide. Hydrazine is used to destroy other pyrimidine rings. These modified nucleotides are resistant to post-labelling.

Diol detection occurs with methyl red in ethanol, boric acid and acetone. These conditions are good for polar sugars; arabinosyl, ribosyl and deoxyribosyl are well handled with PEI in LiCl. Acyclonucleosides are powerful antiviral agents, e.g. acyclovir for herpes. These analogues lack one or more atoms on the pentofuranose ring. Separation strategies can be developed to take advantage of the alterations in the sugar.

Typical specimens from biological sources contain mixtures of purines and pyrimidines. Thin TLC layers (0.1 mm thick) give no elongated spots when used. The separations are fast (10 min) with good efficiency (5000 theoretical plates) at R_F values under 5.5 cm. Ammonium sulfate (0.2–5.0 mol L^{-1}) solutions are used as eluents; other salts (many less ionized than $(NH_2)_4SO_4$) contribute little. pH (borax, acetate, HCl, ammonium) contributes little to separations achieved with ammonium sulfate.

TLC has been used to separate nucleotides from cell culture. TLC gives high resolution, but low load capacity and cumbersome sample-handling procedures. CEL 300 plates and butanol/acetic/water or ethanol/ammonium acetate (pH 5) effect good separations. Colorimetric quantitation is possible with ninhydrin–cadmium. TLC is most effective for nucleotides of relative molecular mass below 4000. Plant viral RNA has been chromatographed with cellulose TLC with *n*-butyric/ammonia/water in one dimension, and ammonium sulfate/sodium acetate/isopropenal in the second. The system easily separates 2′ versus 3′ NMPs. Methylated RNA is separated by 2D-TLC on plates consisting of varying percentages of silica gel/cellulose with acetonitrile (ACN) ethyl acetate/propanol/butanol/water/ammonium hydroxide eluents. Many of these simpler systems line the NMPs in the midline. Pyrimidine/guanine dinucleotides are well separated on PEI with 0.8 LiCl/acetic acid.

An additional challenge to biomedical applications of TLC relates to the separations of cyclic nucleotides from noncyclic phosphates. Alumina TLC and ammonium acetate, pH adjusted with ammonium hydroxide, has been used to effect these separations. 3′-5′-cGMP uses borate impregnated silica in butanol/methanol/ethyl acetate/ammonium hydroxide. Cyclic pyridines/purines are separated on cation exchange layers, pretreated with HCl, as opposed to the popular anion (PEI) systems, with an eluent of 0.05 mol L^{-1} oxalic acid.

The utility of gel electrophoresis for the separation of long chain oligonucleotides has relegated TLC to

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smaller chain species. Intermediate chain oligonucleotides are readily handled by HPLC, but many smaller ones are not. This is the province of TLC. Silica gel TLC has been important in oligomer separations well up to decamers.

tRNA digests can be effectively separated, based on the nucleobase irrespective of adenines. PEI-cellulose in butanol/methanol/water, then formic acid in water, is used. For TLC that is salt-sensitive, PEI plates and 0.5% formic acid in an ascending fashion (occasionally using urea, which reduces smear) are worthwhile. 0.15 mol L^{-1} Li/formate, pH 3.0, achieves separations with as little as 5 DPM after 3 weeks autoradiography. In 2D-TLC systems, one can also add urea/formic acid/pyridine. 2D-TLC is carried out with 22% formic acid in the first dimension and with 0.1 mol L^{-1} formic + pyridine to pH 4.3 in the second dimension. Variation in TLC batches giving different binding capacities and relative primary, secondary and tertiary amine separation were observed but it was felt the results were internally consistent. The best pH is at 4.3, and investigators were successful up to 50 nucleotides.

Avicel cellulose can be used in 2D to 3D with isopropanol/ammonium hydroxide, isobutyric/ammonium hydroxide/EDTA, or ammonium acetate with detection by ethanol/scanning slit UV of the plates. Up to the C_{18} isomer were nicely resolved in a stepwise fashion. Silica gel and ammonium acetate separates up to the C_{12} isomer, and achieves good distinction.

Fingerprint Analyses

In practice all conceivable nucleic acids with altered moieties that form, whether from oxygen stress, aldehydes or other reactive species, cannot be immediately chemically defined on a routine basis. Most investigators in the field of nucleic acid adducts define a particular pattern that is specific to a chemical alteration, mutagen or carcinogen. In ageing research, these are designated 'indigenous' spots. One can employ as much specific chemical characterization as possible, but ultimately may rely on fingerprint analyses. Many of our published figures demonstrate examples of a fingerprint chromatogram (**Figure 1**).

Detectors and Instrumentation

The main detection techniques are colorimetry and visual inspection, zone elution (scraping) for HPLC, spectrometry and GC, or voltammetry, densitometry and radiochemical techniques. More recent methods include computerized radiochemical, laser densitometric and phosphoroimager techniques.

Identification of an unknown analyte requires R_F values that are reproducible to $\pm$ 3%. The geometry of the unknown must conform to the known, under the same chromatographic conditions. Co-chromatography of known and unknowns is always required.

Table 2 lists chromogenic reactions; a great deal of nucleic acid colorimetric information is available from published sources. Common reagents include mercuric acetate (purine and pyrimidines), Ehrlich reagent for *N*-carbamyl amino acids formed after alkali hydrolysis (NaOH), and Pauly reagent for imidazole rings.

Fluorescence remains a standard technique. TLC plates are impregnated with UV fluorescent material at 254 nm (typically zinc silicate). Upon exposure, the nucleic acids absorb at 254 nm and therefore quench, so that they appear black against a blue-green background of fluorescence. The errors in quantification by UV remain high (30%). Some scanning detectors employ UV, which can be applied to TLC plates and gives better quantitative data.

Sensitivity is enhanced by fluorescence techniques but typically these techniques require derivitization (pre- or post-chromatography), which is well described in nucleic acid chemistry.

Fourier transform infrared detection is available for TLC. Many papers had high IR absorbance and were inadequate for direct IR measurement. Papers are now available that allow direct measurement. GC is best employed in conjunction with zone elution, and certainly has application to nucleic acids, though lipids have been more extensively studied.

Mass spectrometry (MS) is readily applied to nucleic acids, but typically after zonal elution to avoid interfering solutes. Present developments in coupled MS-TLC must take into account the sorbent, solvent and analyte – which will not exceed 0.25% w/w based on sample and sorbent. The apparatus requires the ability to extract, elute or volatilize analyte directly from the TLC plate. These instruments will be a boon to the ability to detect and characterize analytes. Investigators have defined nucleic acid photoproducts, radical-induced products, those modified by xenobiotic biotransformation, new and naturally occurring nucleosides especially found in RNA, methylated bases and stable isotopes. Interfaces between MS and liquid chromatography systems have also been extensively investigated.

A large amount of data has been accumulated based on laser densitometry using autoradiographically developed X-ray film from 2D-TLC chromatograms that house the separation of radioactive dNMPs. The exposure times chosen give the ability to label unknowns at high counts, in as little as 2 h, but

typically runs take 24–72 h. Many runs of 96 h to weeks reveal groups of adducts that are reproducible, and many undescribed. Present densitometric techniques can range from a few minutes for unidimensional analyses to 2 h for complete analyses of a 20 cm × 20 cm autoradiogram. Comparison of techniques with direct scintillation counting – the other gold standard – approach r values of 0.99, and similarly correlate with the best quantifying techniques.

Scintillation Counting

The coupling of sensitive (25 DPM above background), rapid (15 min for a 20 cm × 20 cm plate) scintillation counting with computerization represents a major advance in TLC quantification. It allows *in situ* measurement of radioactivity and quantitative reconstruction of the nucleic acids in two or three dimensions. The ready ability to compare TLC plates, generate tables for comparison and rapidly apply statistical or analytical methods by computer is immense. This has revealed quantifiable relationship in nucleic acid and DNA chemistry that were previously quite complicated to examine.

Scintillation counting and densitometry both have very significant advantages and disadvantages. Clearly the ability to quantify a plate immediately after a run and drying makes scintillation counting the 'first-look' method. Yet densitometry allows one to examine, by eye, parts of the chromatogram that may be viewed as background by scintillation counting. These techniques are complementary and will be more extensively used in the future.

Phosphoroimager

Recently, sensitive, rapid phosphoroimagers have been made available that give excellent quantitative data. They employ phosphors able to capture radiation energy from various sources comparable to autoradiography in less than 8% of the time. They not only deliver standard R_F values, but also a mass of quantitative information. The better ability to subtract controls, carry out statistical analyses and enhance minute adducts may revolutionize adduct and analogue detection of nucleic acids.

Immunoassay

This technique, similar to Western blotting, allows colorimetric reactions to occur after binding by antibodies that recognize nucleic acid adducts, and subsequent recognition by enzyme-linked antibodies that cause colorimetric reactions with the application of the appropriate substrate. Little experience is at hand on these techniques, but they increase the specificity of reaction adducts of interest.

Summary and Future Developments

Uniformity in approach is required in TLC chromatography. This article has highlighted information drawn from the wider nucleic acids literature and has referred the reader to new techniques that have been successful in the author's laboratory. These will enhance nucleic acid TLC and the characterization of unknowns, especially where expensive equipment is not available. Greater quantification of data by methods that are now well accepted is needed. The TLC of nucleic acids still remains significantly underused, and it is hoped that this article has offered the investigator a ready source to follow for analytical investigations.

See also: **II/Chromatography: Thin-Layer (Planar):** Densitometry and Image Analysis; Mass Spectrometry; Modes of Development: Conventional; Modes of Development: Forced Flow, Overpressured Layer Chromatography and Centrifugal; Preparative Thin-Layer (Planar) Chromatography; Radioactivity Detection; Spray Reagents. **III/Deoxyribonucleic Acid Profiling:** Capillary Electrophoresis. **Impregnation Techniques: Thin-Layer (Planar) Chromatography. Nucleic Acids:** Extraction; Liquid Chromatography. **Appendix: 2/Essential Guides to Method Development in Two-Dimensional Electrophoresis.**

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Further Reading

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